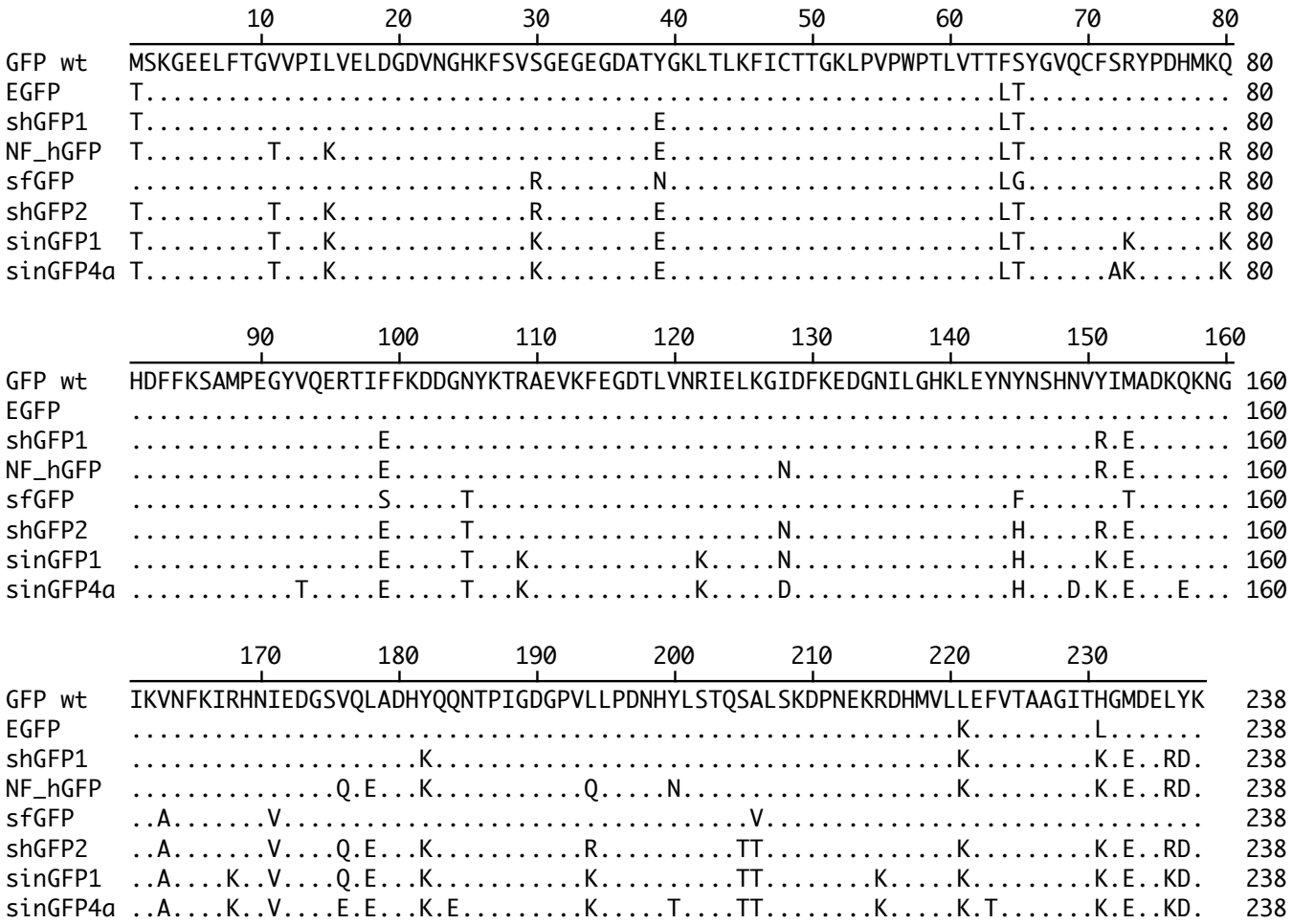


Sequence alignment A: Design of super-inert GFP variants

Figure shows sequence alignment of the key variants. Starting point was EGFP (enhanced GFP; Mut1 in Cormack et al., 1996) carrying an additional dimer-suppressing L221K mutation (Zacharias et al., 2002). shGFP1 (**s**uper**h**ydrophilic GFP 1) combines eight mutations of hydrophobic residues to hydrophilic ones. NF_hGFP was an attempt to make GFP even more hydrophilic, however, this variant was non-fluorescent when expressed at 37°C and nearly non-fluorescent at 18°C. Random mutagenesis and selection identified numerous fluorescence-enhancing mutations, the most hydrophilic of which as well as the dominating N200Y reversion were accepted and combined into the shGFP2 variant, which passed NPCs already ≈ 7-fold slower than EGFP and 2-fold slower than mCherry.

SinGFP1 (super-inert GFP 1) was derived from shGFP2 by 10 R→K exchanges. SinGFP4a was derived (in several steps) from sinGFP1 by increasing its negative charge (by 5 units) as well as by mutating additional exposed hydrophobic residues to hydrophilic ones. SinGFP4a passes NPCs 35-fold slower than EGFP and 10-fold slower than mCherry.

S72A, Y145H, V163A, and S205T are fold-enhancing mutations of non-exposed residues. S30R, N105T, V163A and i171V are superfolder mutations (Pédelacq et al., 2006), while S30K, Y39E, F99E, M153E, Y145H and A206T represent alternative changes at 'superfolder positions'.

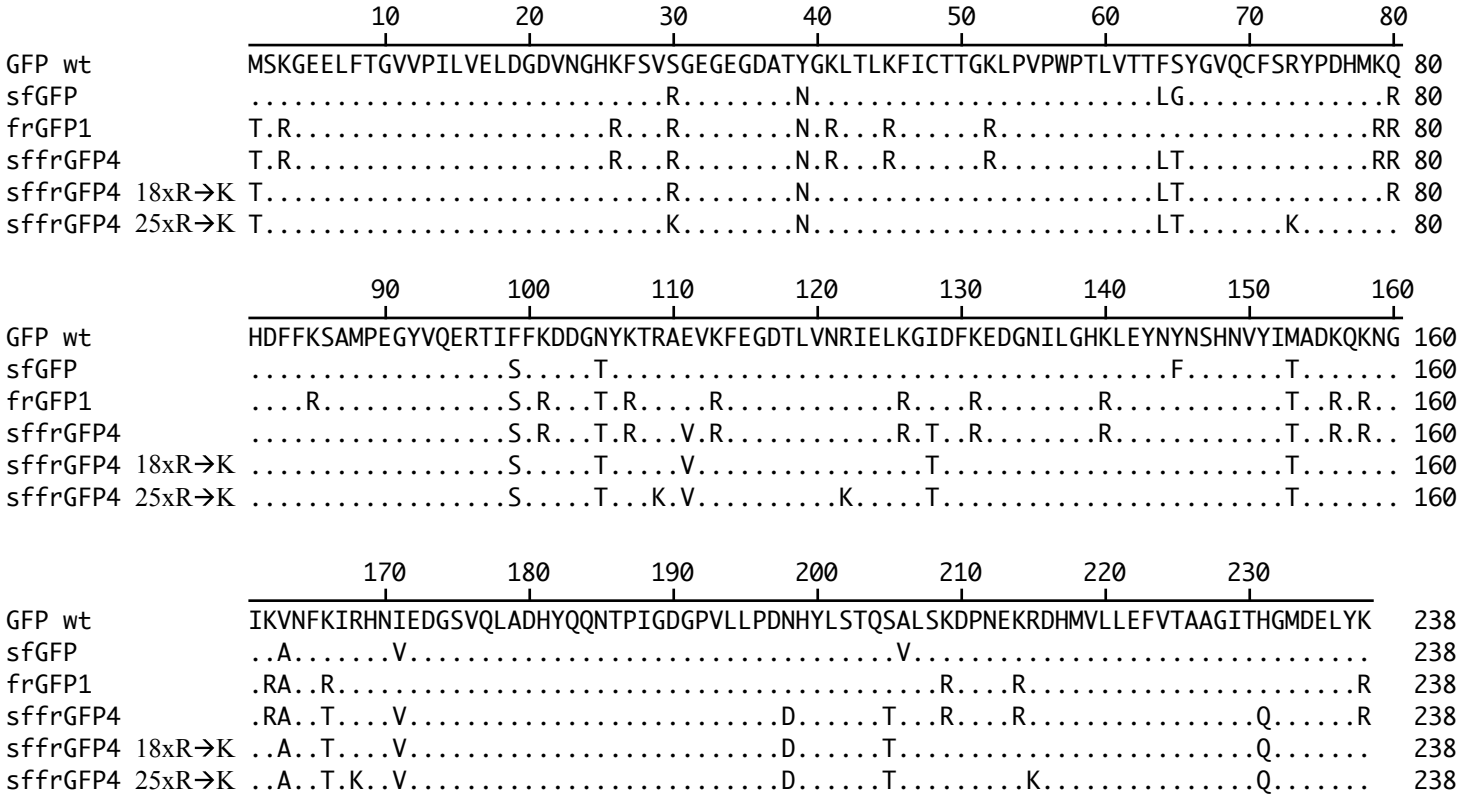
```
                10        20        30        40        50        60        70        80
                |         |         |         |         |         |         |         |
EGFP            TSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQ   80
efGFP           ............................R.........................................H.......   80
efGFP_3W        ............................R.........................................H.......   80
efGFP_5W        ............................R.........W...............................H.......   80
efGFP_8W        ............................R.........W...............................H.......   80
efGFP_8Y        ............................R.........Y...............................H.......   80
efGFP_8F        ............................R.........F...............................H.......   80
efGFP_8L        ............................R.........L...............................H.......   80
efGFP_8i        ............................R.........I...............................H.......   80
efGFP_8M        ............................R.........M...............................H.......   80

                90        100       110       120       130       140       150       160
                |         |         |         |         |         |         |         |
EGFP            HDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNG  160
efGFP           ..................Y.E...A.....................................F.............  160
efGFP_3W        .................W.Y.E...A.....................................F.............  160
efGFP_5W        .................W.Y.E...A.....................................F...W.........  160
efGFP_8W        .................W.Y.E...A.....................................F...W.........  160
efGFP_8Y        .................Y.Y.E...A.....................................F...Y.........  160
efGFP_8F        .................F.Y.E...A.....................................F...F.........  160
efGFP_8L        .................L.Y.E...A.....................................F...L.........  160
efGFP_8i        .................I.Y.E...A.....................................F...I.........  160
efGFP_8M        .................M.Y.E...A.....................................F...M.........  160

                170       180       190       200       210       220       230
                |         |         |         |         |         |         |
EGFP            IKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLKEFVTAAGITLGMDELYK   238
efGFP           ..A..NT...V....E.V.....................N...K.........................G....   238
efGFP_3W        ..AW.NT...V....E.V.....................N.W.K.........................G....   238
efGFP_5W        ..AW.NT...V....E.V.....................N.W.K.........................G....   238
efGFP_8W        ..AW.NT...V....E.V.W...................N.W.K..W.............W........G....   238
efGFP_8Y        ..AY.NT...V....E.V.Y...................N.Y.K..Y.............Y........G....   238
efGFP_8F        ..AF.NT...V....E.V.F...................N.F.K..F.............F........G....   238
efGFP_8L        ..AL.NT...V....E.V.L...................N.L.K..L.............L........G....   238
efGFP_8i        ..AI.NT...V....E.V.I...................N.I.K..I.............I........G....   238
efGFP_8M        ..AM.NT...V....E.V.M...................N.M.K..M.............M........G....   238
```

Sequence alignment B: Engineering of super-hydrophobic GFP variants

The aim was to obtain GFP variants that bind FG domains well enough for a rapid NPC passage. Placing eight ectopic tryptophans onto the GFP surface lead, however, to an essentially non-fluorescent variant. Seven rounds of random mutagenesis and selection identified a set of 15 second-site-mutations that made the GFP_8W variant highly fluorescent. The new scaffold is called efGFP (for enhanced folding of hydrophobic GFPs). Several derivatives were analyzed, namely efGFP with 3, 5, or 8 tryptophans (3W, 5W, 8W) as well as with eight tyrosines (8Y), phenylalanines (8F), leucines (8L), isoleucines (8i) or methionines (8M).

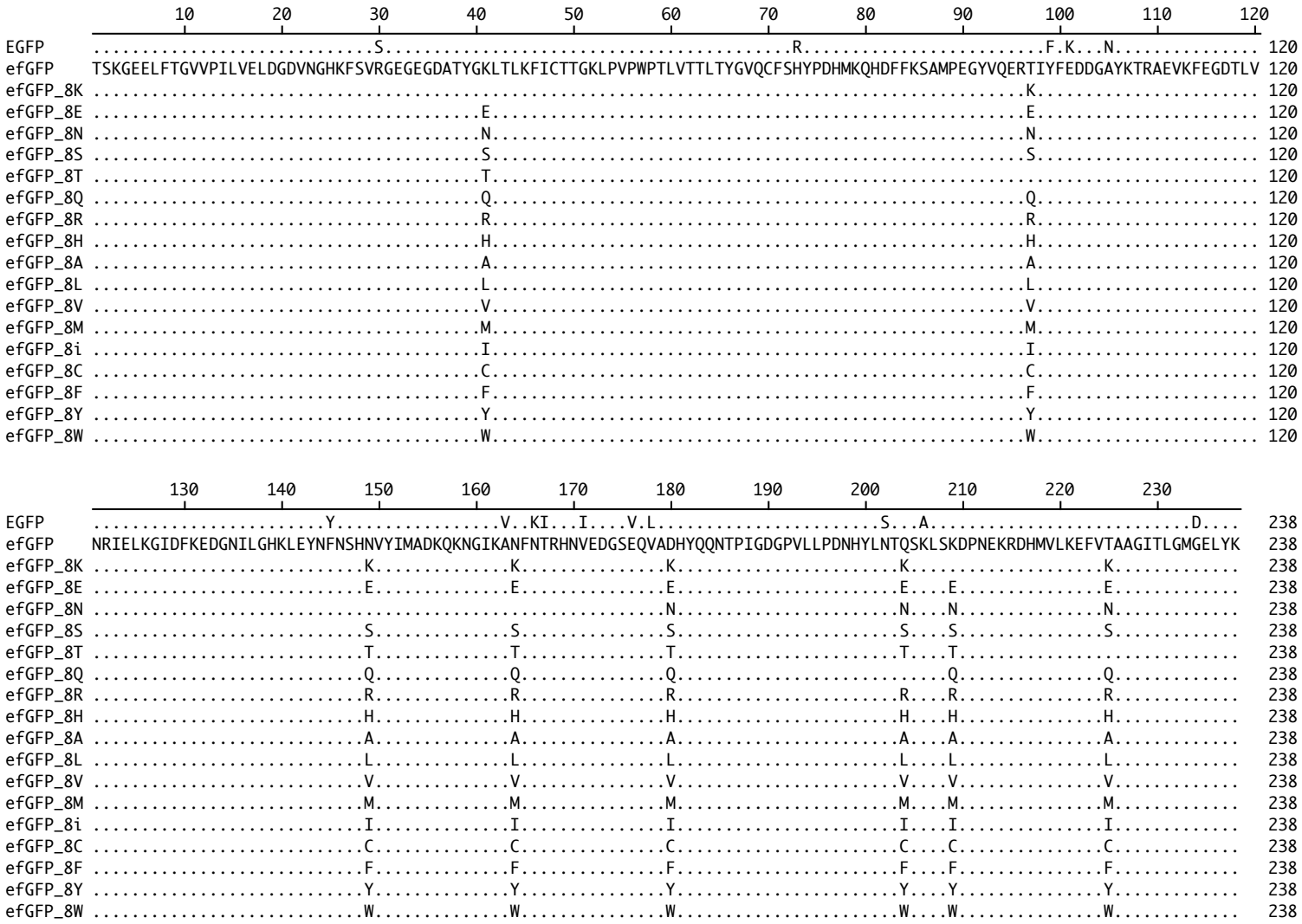


Sequence alignment C: Lys→ Arg substituted GFP variants

For a nuclear transport-unrelated project, we designed a GFP variant, where all lysines had been exchanged to arginines, the rational being to obtain a variant that remains mobile after fixation by aldehydes.

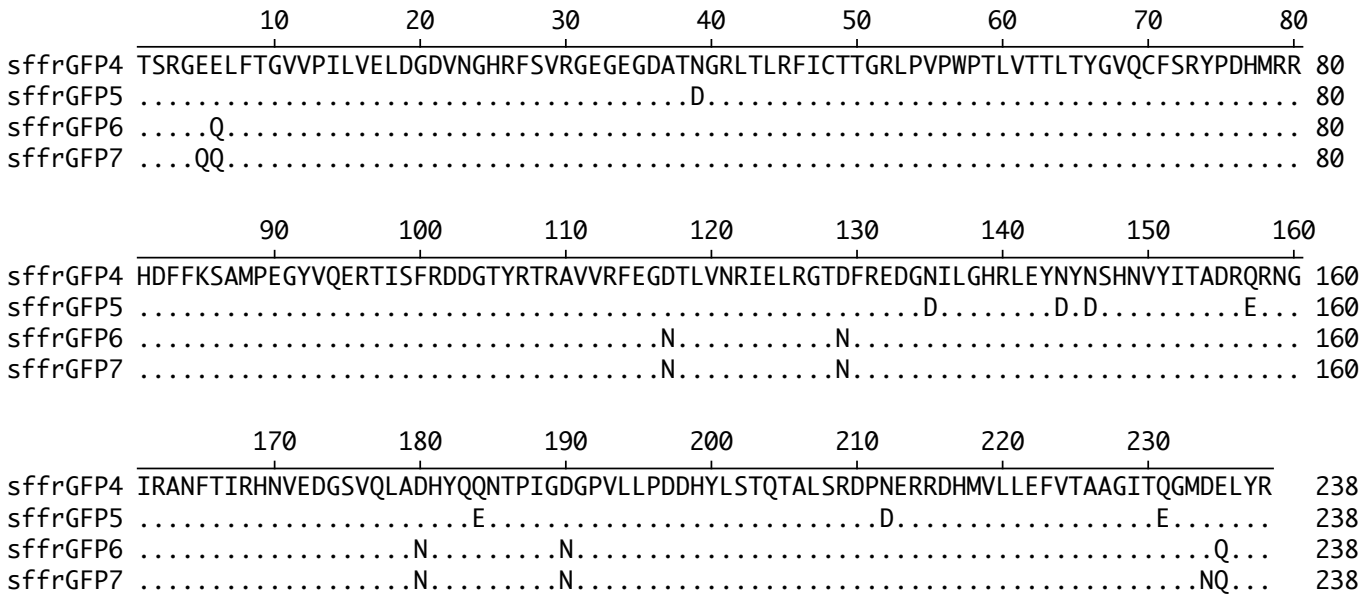
The first variant, frGFP1 (fixation-resistant GFP 1) already contained some fold-enhancing mutations from superfolder GFP (sfGFP; Pédelacq et al., 2006). However, it was non-fluorescent. frGFP1 was then evolved to a highly fluorescent derivative, sffrGFP4 (superfolder fixation-resistant GFP4), which serendipitously turned out to pass NPCs very rapidly.

The alignment also shows sffrGFP4 variants, where the initially introduced Arg-substitutions had been reverted (sffrGFP4 18xR→K) or all surface arginines had been converted to lysines (sffrGFP4 25xR→K). These crossed NPCs ~40 and ~100-fold slower, respectively.



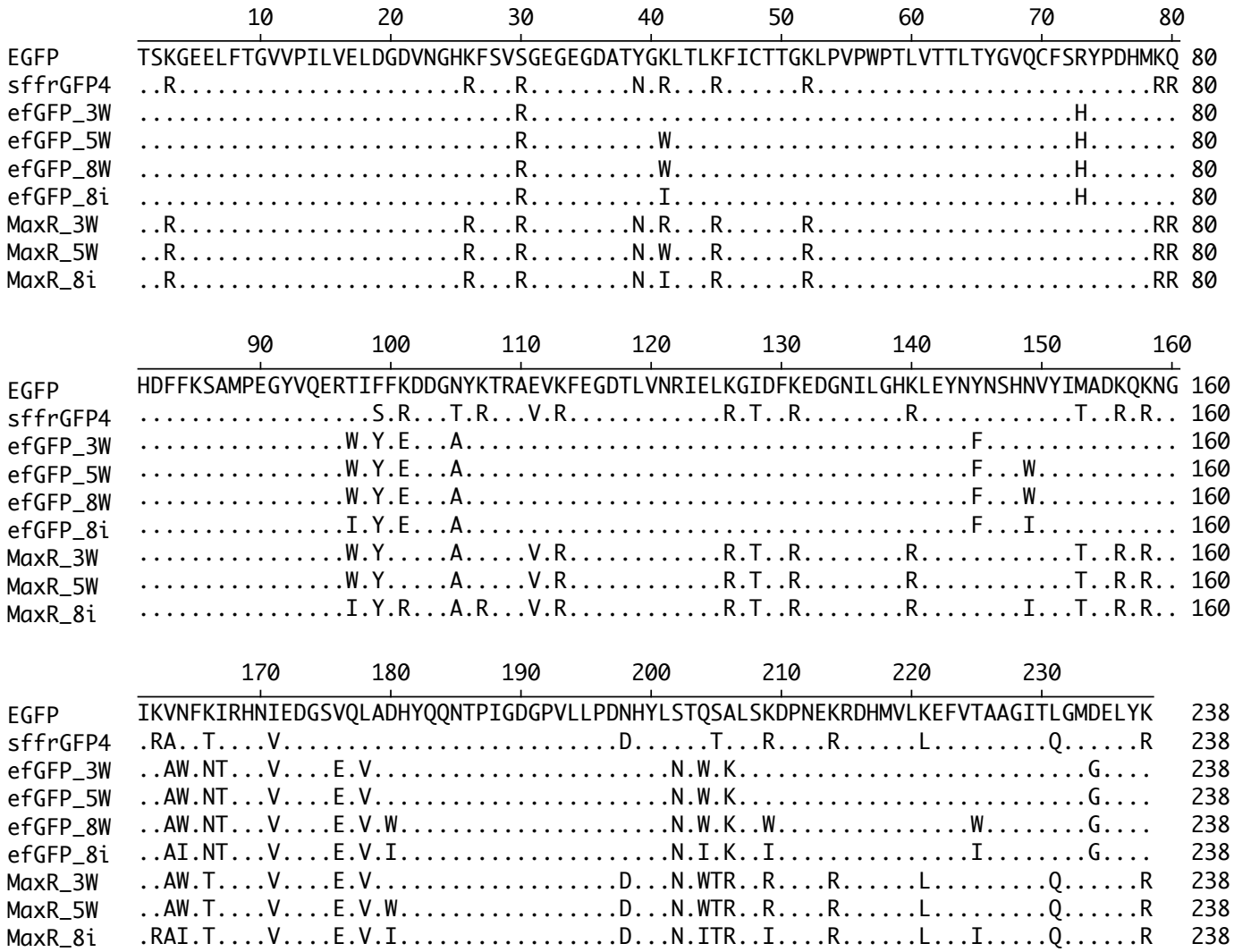
Sequence alignment D: efGFP_8x variants for testing which surface residues impede or facilitate FG phase-entry

Alignment shows the GFP variants tested in Figure 4A and 4B. Each of them displays eight copies of the indicated amino acid side chain.



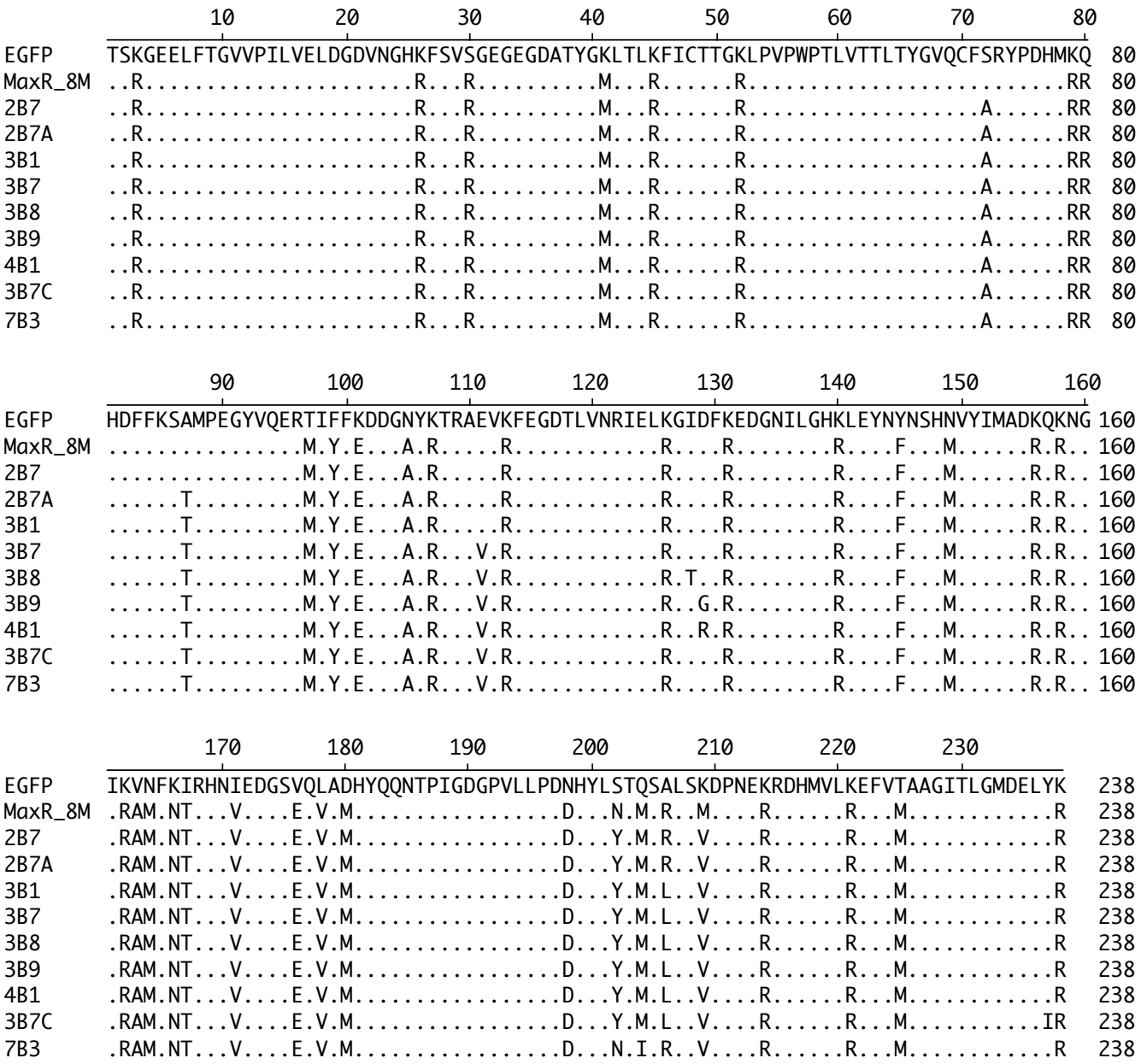
Sequence alignment E: Charge-series based on sffrGFP4

To test the effect of net charge on NPC passage without tampering with exposed arginines or hydrophobic residues, we designed a charge series based on sffrGFP4. All introduced changes where isosteric N-D or Q-E exchanges.



Sequence alignment F: A first generation of GFP^{NTR} variants

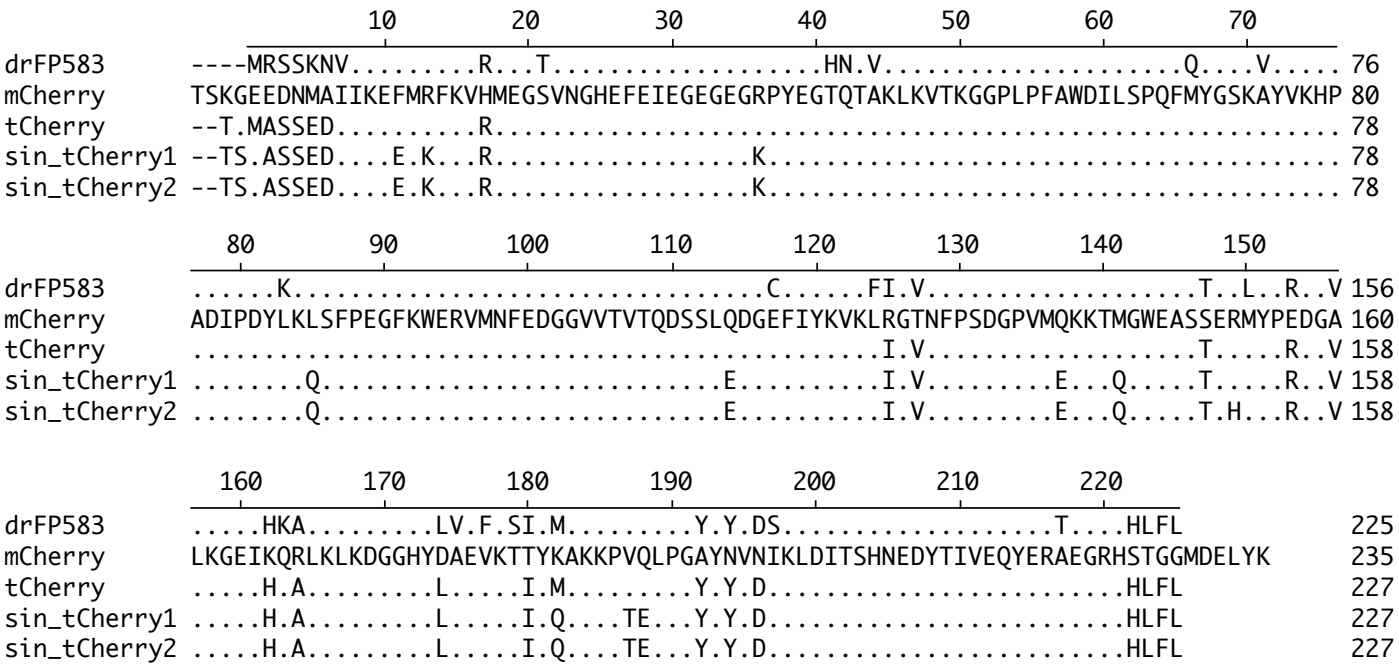
The sffrGFP4 (MaxR) variant and super-hydrophobic variants are shown again for reference. MaxR_3W, MaxR_5W, and MaxR_8i combine the principles of increased surface hydrophobicity with maximal Lys→Arg exchanges. They represent extremely fast NPC-shuttles.



Sequence alignment G: Engineering of GFP^{NTR} variants of high FG-specificity and NPC-passage rate

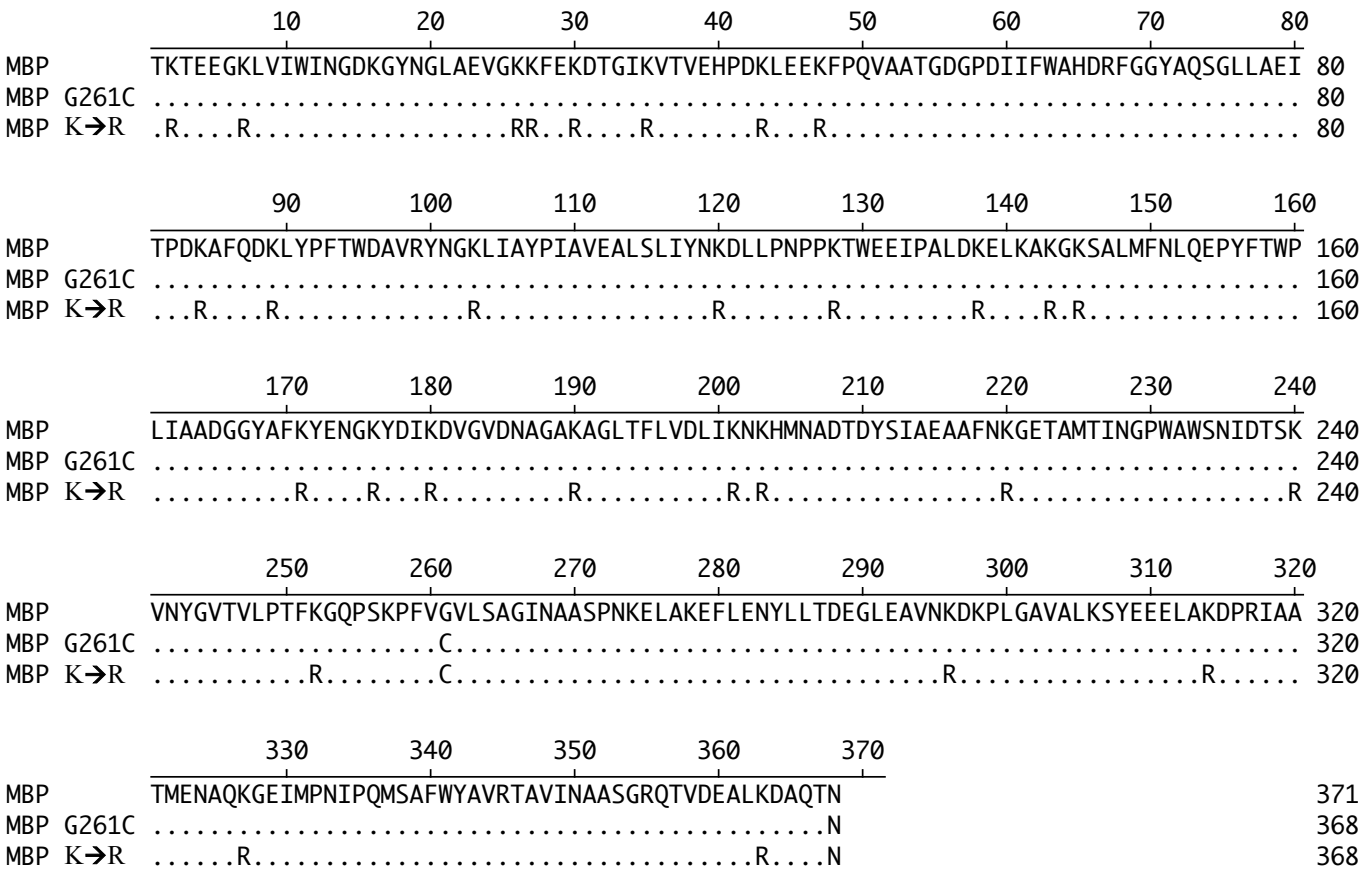
The design principle was to place 8 ectopic (hydrophobic) methionines on the GFP's surface and to replace all exposed lysines by arginines. The essentially non-fluorescent initial MaxR_8M mutant was evolved to highly fluorescent species. 2B7 crosses NPCs considerably faster than NTF2 (see Table 1), but still shows considerable non-specific interactions.

3B1-3B7C are *tetramers* of great FG- and NPC-specificity, with 3B7C being the most selective variant. 7B3 is a re-monomerized version of 3B7 and the fastest NPC-shuttle identified so far, showing less non-specific binding than 2B7.



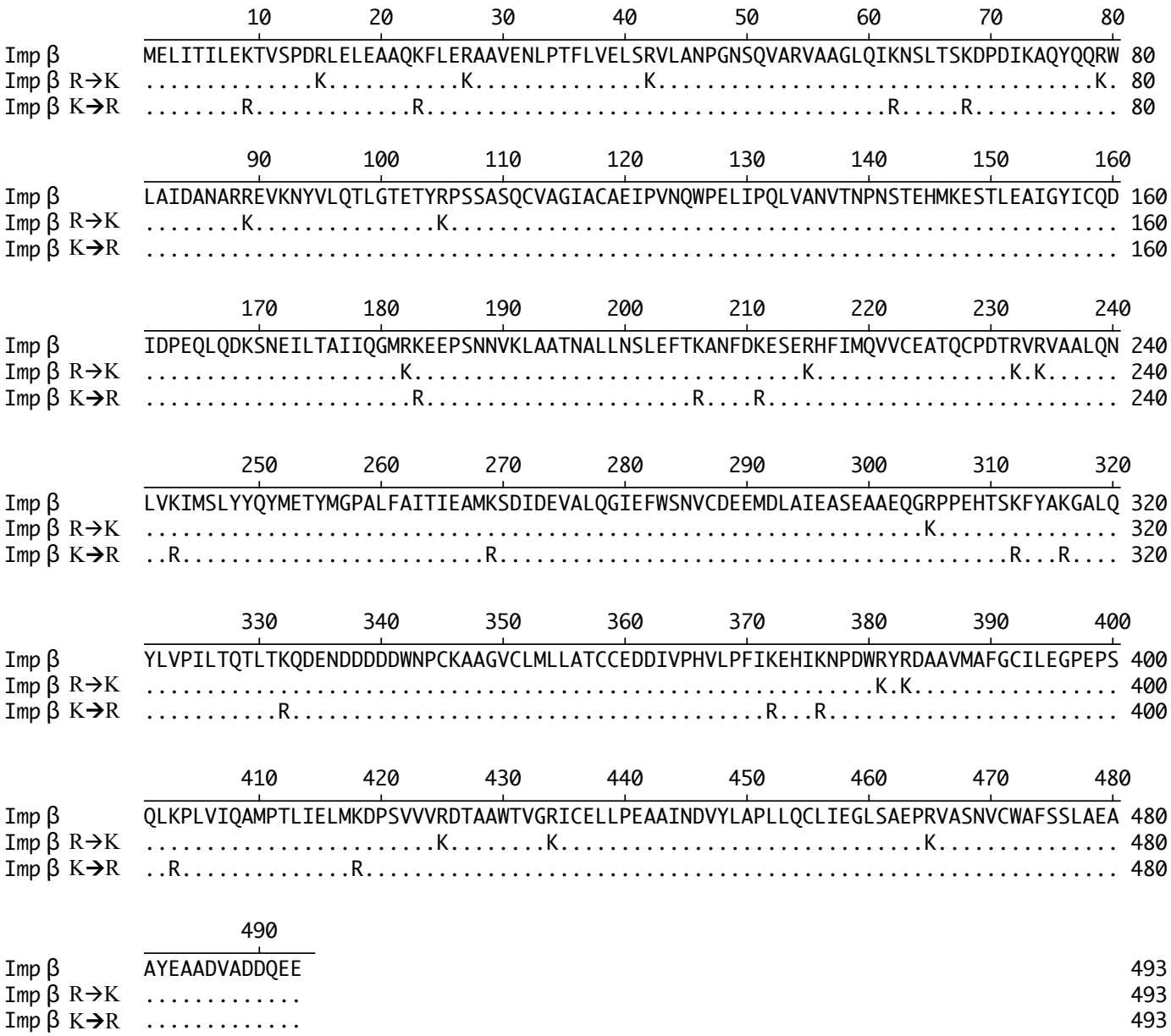
Sequence alignment H: Engineering of a super-inert tetrameric Cherry variant

Starting point was mCherry (Shaner et al., 2004). tCherry is an mCherry derivative, where the tetramerisation interface of the original red fluorescent protein drFP583 had been restored. Sin_tCherry1 is a hydrophilised version of tCherry. Sin_tCherry2 carries an additional R149H exchange that improves the packing between the subunits, increases thermostability, and confers faster fluorophore maturation.



Sequence alignment i: A K→R substituted mutant of the *E. coli* maltose-binding protein MBP, which crosses NPCs 40-fold faster than MBP itself

Alignment shows the implemented substitutions. The G261C mutation creates an attachment site for a maleimide at the base of the maltose-binding groove. An introduced maleimide-fluorophore can thus at least partially be shielded from interactions with FG motifs. MBP K→R contains 29 lysine to arginine exchanges.



Sequence alignment J: K→R and R→K mutations of the human importin β 1-493 fragment

The fragment was used because it expresses better and is more soluble than the full-length version. It also lacks the C-terminal cargo-binding region that would otherwise cause retention inside nuclei when influx is measured without Ran-addition. Figure shows alignment with the corresponding R→K mutant (16 substitutions) and the K→R mutant (16 substitutions).